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Determination of Dihydroqinghaosu in Blood by Pyrolysis Gas Chromatography/Mass Spectrometry

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A sensitive and specific method using packed or capillary column gas chromatography/mass spectrometry (GC/MS) has been developed for the quantitation of the major active metabolite dihydroqinghaosu (DQHS) of the antimalarial drug artesunic acid in blood. Quantification is achieved with an internal standard method, petroleum ether or *n*-butyl chloride extraction of blood and gas chromatography/mass spectrometry analysis of the extract with selected ion monitoring. Since DQHS is thermally unstable, the method is based on the observations that at high injector temperature and specific GC conditions, DQHS is pyrolyzed to (2*S*,3*R*,6*R*)-2-(3-oxobutyl)-3-methyl-6-[(*R*)-2-propanal]cyclohexanone. Quantitation of this cyclohexanone pyrolysis product by GC/MS with selected ion monitoring precisely and accurately reflects DQHS blood levels and calibration curves are linear in the range of 10–1000 ng/mL. The method is currently being used for pharmacokinetic and metabolism studies in animals.

Over the last 12 years, the active antimalarial principal of the Chinese herb qinghao (*Artemisia annua L.*) has been isolated and chemically characterized (*1–4*). This compound, a unique 15-carbon sesquiterpene lactone endoperoxide (Figure 1) named qinghaosu (QHS) or artemisinin, and its synthetic derivatives have been shown to be more effective than chloroquine against the erythrocytic stages of plasmodia; their parasiticidal action is faster, and they are equally effective against chloroquine-resistant strains of parasites (*5*). In recent years, over 2000 cases of vivax or falciparum malaria have been treated with QHS or its derivatives in China with excellent results (*6*). No toxic side effects were observed in these studies (*6*), and it has been reported that QHS and its derivatives are superior to chloroquine in chemotherapeutic index, margin of safety, and side effects (*7*). Furthermore, it has been shown that QHS and its derivatives are extremely effective in the treatment of cerebral malaria (*8*). A comprehensive review on the chemistry and some of the pharmacological properties of artemisinin and its analogues was recently published by Klayman (*9*).

Previous studies on the pharmacokinetics of artesunic acid (ARTA) (Figure 1) after intravenous administration of the sodium salt suggested that ARTA is rapidly hydrolyzed to dihydroqinghaosu (DQHS) in rats (*10*), therefore, DQHS was measured rather than ARTA. DQHS was measured by thin-layer chromatography (TLC), staining with *p*-(dimethylamino)benzaldehyde. The sensitivity of the method was reported to be 0.5 μg (*10*). Considering the low dose of ARTA administered to humans, the poor sensitivity and lack of specificity of the TLC method, and the fact that little is known about the pharmacokinetics of ARTA and DQHS in the therapeutic range, our laboratory sought to develop new specific and sensitive methodologies to accurately measure DQHS in blood in the nanogram per milliliter range. Since ARTA and DQHS have no UV–vis or fluorescence properties, our efforts were focused on developing suitable gas chromatography/mass spectrometry (GC/MS) techniques for the quantitation of DQHS in blood. Initial experiments demonstrated that DQHS is thermally unstable and is degraded to two major and a number of minor compounds during GC/MS. The structural identification of these two major pyrolysis products has been determined (*11*) (see Figure 2). Other experiments also showed that the relative formation of these two major pyrolysis products is dependent on the conditions of chromatography and that suitable chromatographic conditions can be utilized such that only one major pyrolysis product of DQHS is formed during GC/MS (>90%). In this paper, the results on the development and validation of packed and capillary column gas chromatographic methodologies are described in which DQHS is pyrolyzed in the GC injector to one major degradation product such that accurate and precise quantitation of DQHS in blood can be achieved in the low nanogram per milliliter range. We also demonstrate the utility of the developed methods in measuring blood levels of DQHS after in vivo administration of ARTA to rabbits and dogs.

EXPERIMENTAL SECTION

Chemicals and Supplies. Artesunic acid and dihydroqinghaosu were provided by the Institute of Chinese Materia Medica, Academy of Traditional Chinese Medicine, Beijing, China. Cedrol and triphenylmethanol (internal standards) were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Eastman Organic Chemicals (Rochester, NY), respectively. Petroleum ether was purchased from Mallinckrodt, Inc. (Paris, KY), and ethyl acetate, *n*-butyl chloride, and methyl *tert*-butyl ether were purchased from Burdick and Jackson (Muskegon, MI). Fused silica capillary columns (15-m SPB-1 and 15-m SPB-5, 0.25-μm film thickness, 0.25 mm i.d.) and 3% OV-3, 3% OV-17, or 3% SP-2100 on 100/120 Supelcoport for packed column studies were purchased from Supelco, Inc. (Bellafonte, PA). The dry solids injector used for capillary column studies was purchased from Allen Scientific (Boulder, CO).

Gas Chromatography/Mass Spectrometry. GC/MS was performed with a Varian Vista gas chromatograph (GC) (Palo Alto, CA) interfaced to a Nermag R10-10C quadrupole mass spectrometer (Delsi Nermag, Fairfield, NJ). The mass spectrometer was interfaced to an INCOS data system (Finnigan Corp., Sunnyvale, CA). Packed column GC was performed with 6 ft × 2 mm i.d. silanized glass columns using either helium or methane as carrier gas at a flow rate of 20 mL/min (measured at atmospheric pressure at column outlet). A 1000 L/s diffusion pump on the source region of this instrument permits packed column GC without carrier gas splitting; therefore, the effluent from the GC was plumbed directly to the ion source with a 2 ft length of fused silica tubing. For capillary column GC, split/splitless, on-column, or dry solids injection techniques were utilized with helium as carrier gas at a flow rate of 40 cm/s. The outlet of the capillary column was plumbed directly to the ion source. EI or CI mass spectrometry was performed at 70 and 90 eV, respectively, and 130 °C source temperature. Methane or isobutane were used

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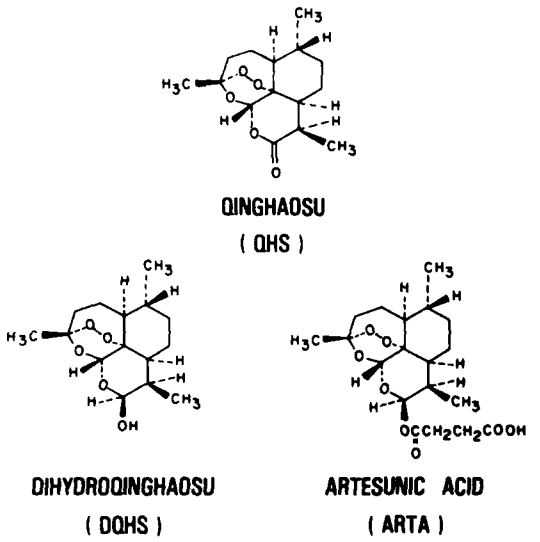

Figure 1. Structures of qinghaosu (QHS), dihydroqinghaosu (DQHS), and artesunic acid (ARTA).

as reagent gases for CI at a source pressure of 0.25 Torr for capillary and 0.4 Torr for packed column gas chromatography.

Quantitation of DQHS by GC/MS with Selected Ion Monitoring. Detection and quantitation of DQHS and internal standard were achieved by monitoring the intensity of m/z 221.3 ± 0.5 amu under methane CI conditions for method 1 or 221.3 ± 0.5, 239.3 ± 0.5, and 243.3 ± 0.5 amu for method 2. Packed column GC was performed with a 6 ft × 2 mm i.d. silanized glass column packed with 3% OV-3 on 100/120 Supelcoport (Supelco). Conditions of GC were as follows: injector, 320 °C; interface, 240 °C; carrier gas, CH_4 (20 mL/min). A temperature gradient run from 160 to 240 °C at 10 °C/min was utilized to elute internal standard (cedrol, method 1) (retention time, 3.2 min) and the cyclohexanone pyrolysis product of DQHS (retention time 5.5 min). Capillary column GC was performed with a 15-m SPB-5 fused silica column connected to a dry solids falling needle injector. Conditions of GC were as follows: injector, 320 °C; carrier gas, He (40 cm/s); interface, 240 °C; system was run from 100 to 200 °C at 10 °C/min to elute compounds of interest.

Quantitation of DQHS in blood was achieved by using peak height ratios of the cyclohexanone pyrolysis product of DQHS to internal standard (cedrol or triphenylmethanol). For each analysis, a standard curve was generated by adding known and varying amounts of DQHS and a constant amount of the internal standard (500 or 1000 ng for high range and 100 ng for low range, method 1; 20 ng, method 2). Spiked samples were treated as unknowns to evaluate the precision and accuracy of the methods.

Standard Solutions. All standard stock solutions of DQHS (10 ng/μL, 1 ng/μL, 100 pg/μL) and internal standard, cedrol or triphenylmethanol (10, 1, 0.1 ng/μL), were prepared fresh daily and dissolved in HPLC grade methyl *tert*-butyl ether or ethyl acetate.

Extraction Procedure for DQHS. *Method 1.* To disposable glass extraction tubes (16 × 125 mm) previously washed with 3 mL of methyl *tert*-butyl ether, were added various amounts of DQHS and cedrol. Solvent was evaporated with a Buchler vortex evaporator and then 1 mL of heparinized (10 IU/mL) human blood was added to each sample. Samples were vortexed and then kept at room temperature for 15–20 min. Four milliliters of petroleum ether was added to each sample; samples were then vortexed for 30 s each and centrifuged at 2500g for 5 min. The upper organic phase was removed and transferred to clean extraction tubes. An additional 4 mL of petroleum ether was added to the aqueous sample, and the above procedure was repeated. The petroleum ether extracts were combined and evaporated to dryness at room temperature with a Buchler vortex evaporator. The dry residues were reconstituted in 100 μL of ethyl acetate for GC/MS analysis with selected ion monitoring (GC/SIM). By use of external standards, extraction recoveries of DQHS and cedrol were 20–60% and 60–80%, respectively.

The pronounced variability in the extraction efficiency of DQHS was due to decreased recovery at low blood levels of DQHS (less than 50 ng/mL), whereas recoveries in the 100–1000 ng/mL range were 55–65%. This dependence of the extraction efficiency on the amount of DQHS present in blood was always consistent and was corrected for by the internal standards ratio method. As shown in Tables I and II, standard curves with this method showed good correlation to a linear equation.

During the development of the extraction methodologies, many different types of solvents (*tert*-butyl ether, diethyl ether, methylene chloride, ethyl acetate, hexane) and mixtures of these solvents were tested in an attempt to improve the extraction efficiency of the method. Initial experiments showed that these solvents were found to be unsatisfactory because of poor recovery or because the extracts were heavily contaminated with endogenous blood constituents. Additional experiments designed to study the utility of protein precipitation followed by liquid–liquid or liquid–solid extraction also failed for the same reasons.

Although the extraction efficiency with petroleum ether is poor, we chose to validate the method using this solvent for extraction, in order to initiate animal pharmacokinetic studies and, at the same time, continue studies to improve the specificity and recovery of the initial extraction step.

Additional detailed observations of the characteristics of the method and performance of the procedures by different analysts demonstrated that the internal standard, cedrol, slowly sublimed from the dry solids injector needle (60% in 30 min) and that linear standard curves and reproducible results with this internal standard could only be obtained if the sample was injected shortly after solvent evaporation from the needle (less than 2 min).

Method 2. This extraction procedure is identical with that described in method 1 except that triphenylmethanol was used as the internal standard, and the extraction was performed with *n*-butyl chloride rather than petroleum ether. Recoveries for DQHS and triphenylmethanol were 76 ± 12% and 100 ± 15%, respectively, with this method.

Since a major pyrolysis product of ARTA on GC is the same cyclohexanone pyrolysis product formed from DQHS, some experiments were performed in which blood was spiked with ARTA (1 μg/mL) and cedrol or ARTA and triphenylmethanol, and then samples were extracted with petroleum ether or *n*-butyl chloride and analyzed as described above. The results demonstrated that less than 0.1% of the spiked ARTA was extracted and measured as the cyclohexanone pyrolysis product. These results prove that the presence of ARTA in blood does not affect the quantitation of DQHS, since ARTA is not extracted from blood with petroleum ether or *n*-butyl chloride under these conditions.

In Vivo Infusion and Sampling Methodologies. All animal studies were conducted by using approved protocols in compliance with all DoD regulations and the NIH Publication, 85-23, *Guide for the Care and Use of Laboratory Animals*. Animals were housed in standard cages and given food (Rabbit Chow, Dog Lab Diet, Ralston Purina, St. Louis, MO) and tap water ad libitum. All animals were in a fed state at the time of study. Initial experiments to determine the feasibility and utility of the developed methodologies were with New Zealand white rabbits, male, weighing 2–3 kg. Each animal was restrained in a nalgene restraint cage during the 2-h study period. Repeated blood sampling was accomplished by using the medial artery of one ear by the method of Paulsen and Valentine (*12*). Drug at a dose of 5 mg/kg was administered as a bolus injection into the marginal ear vein of the opposite ear. The drug, the sodium salt of ARTA, was prepared by dissolving ARTA in sterile 3.5% sodium bicarbonate (Gibco Laboratories, Grand Island, NY) at a concentration of 200 mg/mL. Healthy, manually restrained, and awake male beagle dogs (9.3 and 10.4 kg) were also used to study DQHS pharmacokinetics after intravenous administration of ARTA. An indwelling pediatric, disposable intravenous catheter with a 23-gauge needle was aseptically placed into the cephalic vein of one of the forelegs for blood sampling. Patency of this catheter was maintained by instilling a volume of saline containing 30 units/mL of heparin.

Blood samples were drawn into heparinized-rinsed (30 units/mL of saline) 3-cm^3 syringes after withdrawing the heparinized saline and approximately a half milliliter of blood into a separate syringe. The cephalic vein of the opposite leg was used to ad-

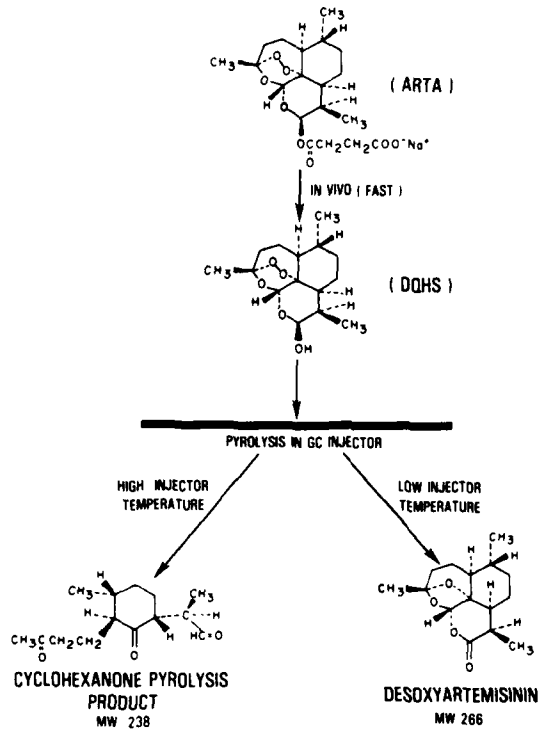

Figure 2. Reaction scheme of in vivo hydrolysis of sodium artensunate to dihydroqinghaosu and pyrolysis reactions in GC. Low injector temperature (180 °C) causes primarily desoxyartemisinin formation whereas high injector temperature (320–350 °C) causes cyclohexanone pyrolysis product formation.

minister the drug at a dose of 5 mg/kg. The sodium salt of ARTA was prepared as described above in the rabbit studies; however this solution was then diluted with 5% glucose solution such that the final solution was isotonic. This solution was administered as a bolus injection into the opposite leg as previously mentioned.

Heparinized blood samples were frozen immediately after sampling with a dry ice–acetone bath and kept at −70 °C for the duration of the experiment. Samples were then thawed at room temperature and extracted as described above. Blood concentration time data were analyzed by using model equations with an iterative nonlinear curve fitting computer program with a nonweighted least-squares criterion of fit (*13*).

RESULTS AND DISCUSSION

Previous reports have suggested (*10*) and information obtained in this laboratory with HPLC methodologies using reductive electrochemical detection (*14*) have demonstrated that ARTA is rapidly hydrolyzed to DQHS in vivo as depicted in Figure 2. DQHS is considered to be the active antimalarial compound (*15*, *16*), and ARTA is utilized only for purposes of water solubility and intravenous administration and should be considered as a prodrug of DQHS. Because of this, we focused on developing accurate and precise methodologies to quantitate DQHS in blood. However, DQHS is thermally unstable and degrades to two major pyrolysis products during GC. Figure 2 shows the structures of these two compounds (cyclohexanone pyrolysis product (2*S*,3*R*,6*R*)-2-(3-oxobutyl)-3-methyl-6-[(*R*)-2-propanal]cyclohexanone and desoxyartemisinin) which were recently characterized structurally as a result of these observations (*11*). Early studies also showed that the pyrolysis products formed and the ratios were quite variable, and dependent on injector type, injector temperature, column packing, and duration of run. More specifically, the use of an on-column injector (50–160 °C at 10 °C/min) with a 6-meter SPB-1 fused silica column 50–160 °C ballistically, then 10 °C/min to 180 °C) caused primarily

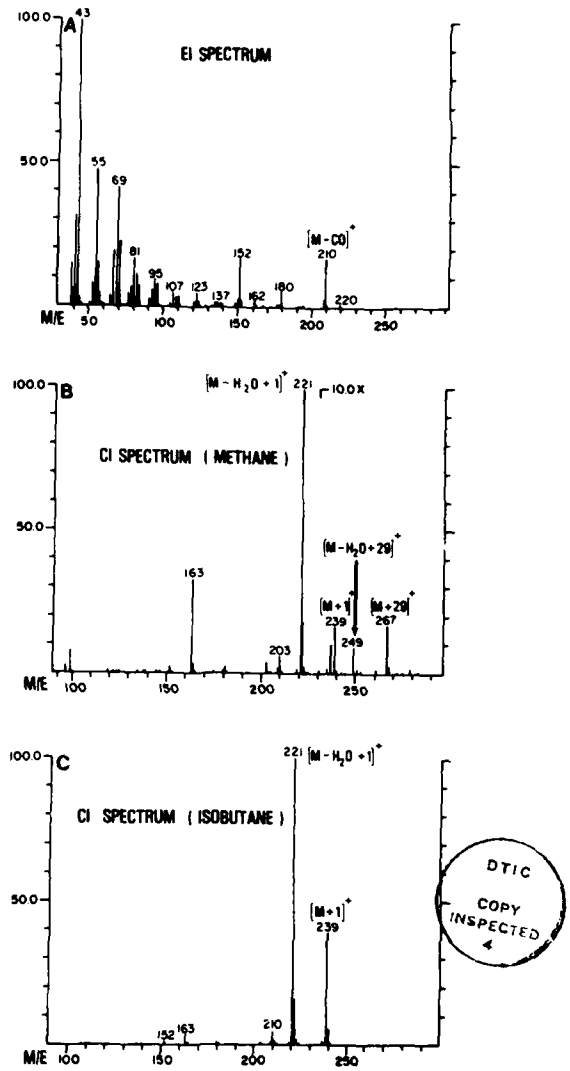

Figure 3. EI and CI mass spectra of the cyclohexanone pyrolysis product of DQHS. Spectra were obtained by injecting DQHS on a 15-m SPB-5 capillary column with the dry solids injector: injector, 320 °C; (A) EI spectrum, (B) CI spectrum, methane, (C) CI spectrum, isobutane.

desoxyartemisinin formation (80%) and only 20% of the cyclohexanone pyrolysis product. Furthermore, it was observed that the cyclohexanone pyrolysis product formation could not be eliminated even under very mild conditions. However, extremely high injector temperature (300–400 °C) completely eliminates the formation of desoxyartemisinin on 3% SP2100, 3% OV-3 or 3% OV-17. The same findings were obtained by capillary column GC; however elimination of desoxyartemisinin formation (<2.5%) was obtained only when the dry solids injector was used. These results suggested that quantitation of DQHS in blood by GC/MS is feasible if DQHS is first pyrolyzed in the GC injector to the cyclohexanone pyrolysis product and quantitation is performed on this compound.

Figure 3 shows the EI and CI mass spectra of the cyclohexanone pyrolysis product obtained from injection of DQHS and some structural assignments of major ions. No molecular weight information is obtained from the EI spectrum; however some structural information is present. The methane CI spectrum shows an additional methane adduct ion at m/z 249 and is probably due to dehydration before chemical ionization ($[M - H_2O + C_2H_5]^+$); i.e., the cyclohexanone pyrolysis product

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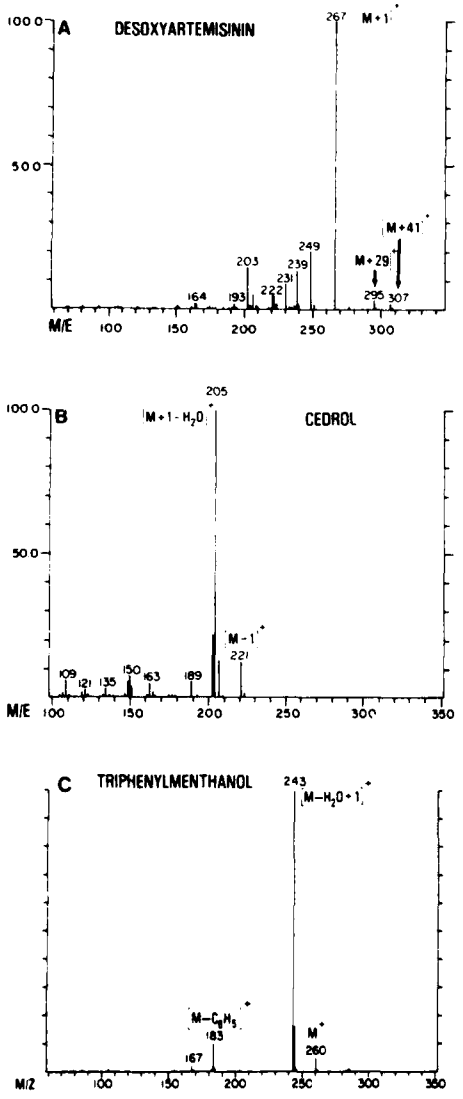

Figure 4. CI mass spectra of desoxyartemisinin (methane), and internal standards cedrol (methane) and triphenylmethanol (isobutane). (A) Desoxyartemisinin, obtained by injecting DQHS on a 15-m SPB-5 capillary column; injector, 180 °C. (B) Cedrol, obtained by using identical conditions as described in Figure 3. (C) Triphenylmethanol.

and a dehydrated cyclohexanone pyrolysis product are present in the ion source at the same time. These results demonstrated that methane or isobutane CI and selected ion monitoring of m/z 221 could be used to quantitate the cyclohexane pyrolysis product of DQHS.

Figure 4 shows the methane CI spectra of desoxyartemisinin, cedrol, and triphenylmethanol. The methane CI spectrum of cedrol is shown in Figure 4B, with base peak at m/z 205 due to dehydration of the tertiary alcohol of the molecule. Pseudo molecular ion at m/z 221 $[M - 1]^+$ is also present. Since this ion (m/z 221) is also the base peak ion of the cyclohexanone pyrolysis product spectrum, it appeared that quantitation of both cedrol and the cyclohexanone pyrolysis product of DQHS could be performed by GC/SIM by monitoring m/z 221 (method 1). Figure 5 shows chromatograms of the intensity at m/z 221, 239, and 243 of extracts of blood spiked with 20, 50, and 0 ng/mL of DQHS, respectively, with sample cleanup performed as described for method 2. One percent of the total extract was injected on column, representing approximately 150 and 375 pg on column for DQHS and 200 pg for triphenylmethanol. Samples in which

Table I. Precision and Accuracy Data for Analysis of DQHS in Human Blood by Packed Column GC/SIM[a]

amt added, ng/mL	amt measd (ng/mL ± std dev)	rel std dev, %	accuracy (% error)	n
100	106 ± 10	9.4	+6	20
400	411 ± 38	9.1	+3	20

[a] Data represent a compilation of five separate experiments using method 1. Samples for standard curves contained 500 ng of cedrol (internal standard) and 0, 100, 200, 300, 500, 750, or 1000 ng of DQHS. Mean correlation coefficients (r^2) for five standard curves were 0.992 ± 0.005. Intraday coefficients of variation ranged from 2 to 12% and 2 to 7% for 100 and 400 ng/mL, respectively. 5% of the total extract was injected on column for both standards and samples.

Table II. Precision and Accuracy Data for Analysis of DQHS in Human Blood by Capillary Column GC/SIM[a]

amt added, ng/mL	amt measd (ng/mL ± std dev)	rel std dev, %	accuracy (% error)	n
20[b]	22.6 ± 1.4	6.2	+13	16
50[b]	49.1 ± 5.4	11.0	-1.9	16
100[c]	99.4 ± 4.2	4.2	-0.6	20
400[c]	386 ± 29	7.5	-3.6	20

[a] Data represent a compilation of ten separate experiments using method 1 with $n = 4$ for each experiment. 5% of the total extract was injected on column for both standards and samples. [b] Samples for standard curves contained 100 ng of cedrol and 0, 10, 20, 30, 50, or 100 ng of DQHS. Mean correlation coefficients (r^2) for four standard curves were 0.980 ± 0.016. Intraday coefficients of variation ranged from 9 to 18% and 2 to 13% for 20 and 50 ng/mL, respectively. [c] Samples for standard curves contained 1000 ng of cedrol and 0, 50, 100, 200, 300, 500, or 750 ng of DQHS. Mean correlation coefficients (r^2) for five standard curves were 0.976 ± 0.020. Intraday coefficients of variation ranged from 4 to 10% and 4 to 12% for 100 and 400 ng/mL, respectively.

Table III. Precision and Accuracy Data for Analysis of DQHS in Human Blood by Capillary Column GC/SIM[a,b]

amt added, ng/mL	amt measd (ng/mL ± std dev)	rel std dev, %	accuracy (% error)	n
20	19.8 ± 0.9	4.8	1.2	16
50	49.8 ± 3.6	7.2	0.3	16

[a] Data represent a compilation of four separate experiments using method 2 with $n = 4$ for each experiment. 1% of the total extract was injected on column for both standards and samples. [b] Samples for standard curves contained 20 ng of triphenylmethanol and 0, 10, 20, 30, 50, 100, and 500 ng of DQHS. Mean correlation coefficients were 0.980 ± 0.012. Intraday coefficients of variation ranged from 5 to 20% and 2 to 13% for 20 and 50 ng/mL, respectively.

DQHS, triphenylmethanol, or both compounds were omitted, demonstrated that no detectable interferences eluting in the regions of interest were present using either the capillary or packed column method (Figure 5C).

Results from the validation studies of the method for both packed and capillary column procedures are shown in Tables I–III. By use of the packed column procedure, excellent results (less than 10% relative standard deviation and less than 10% error in accuracy) were achieved at 100 and 400 ng/mL (Table I). Validation of the capillary column procedure at 20, 50, 100, and 400 ng/mL was also achieved with good results (Table II) by using method 1. Validation studies using method 2 with the capillary column procedure are shown in Table III. Excellent precision and accuracy data were obtained by using this method as well. Furthermore, much less contamination was present using n-butyl chloride for extraction than with petroleum ether extraction. Since ex-

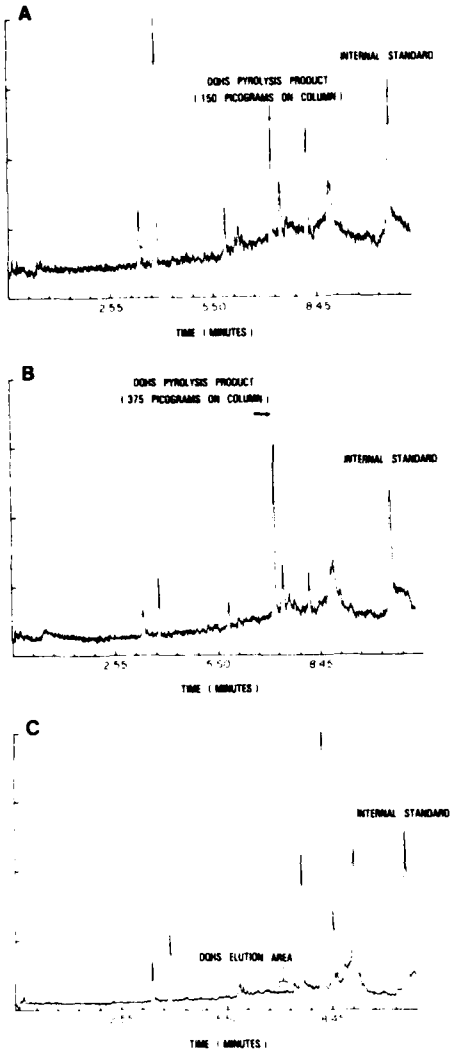

Figure 5. Selected ion chromatograms of human blood spiked with DQHS and internal standard, triphenylmethanol: (A) 20 ng/mL, 1% of the total extract; (B) 50 ng/mL, 1% of the total extract; (C) blank, 5% of the total extract.

traction efficiencies were higher with *n*-butyl chloride extraction, only 1% of the total extract on column was required for an adequate signal-to-noise ratio at 10 ng/mL. This also resulted in a much longer column lifetime.

Figure 6A shows the blood levels of DQHS measured using method 1 after intravenous administration of 5 mg/kg of the sodium salt of ARTA to a rabbit, and a plot of the computerized nonlinear regression best fit of the data. Blood levels ranged from approximately 400 ng/mL at 8 min postdosing and decreased to 8 ng/mL at 110 min. Excellent fits of the data were obtained with a two-compartment open model consisting of the sum of two exponential terms (*13*). Ninety-nine percent of the total area under the curve (346 ng·mL^{-1}·h (0–110 min vs 0 to ∞ [trapezoidal rule]) was characterized during the sampling period with a terminal elimination half-life of 17.7 min. In a second identical experiment in the same rabbit (not shown), total area under the curve was 483 ng·mL^{-1}·h with a terminal elimination half-life of 19.1 min. Figure 6B shows blood levels of DQHS measured using method 2 after intravenous administration of 5 mg/kg of the sodium salt of ARTA of two beagle dogs and a plot of the computerized nonlinear regression fit of the data. Area under the curve and terminal elimination half-life were calculated

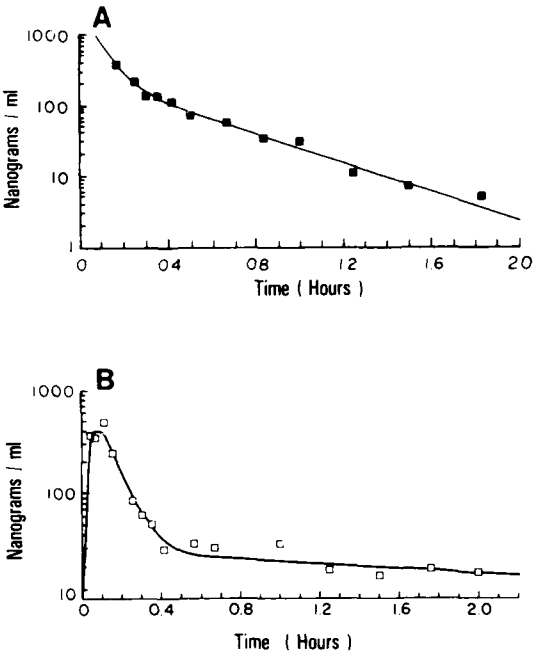

Figure 6. Blood concentration/time data for DQHS after intravenous administration (5 mg/kg) of the sodium salt of ARTA in a rabbit (A) and a dog (B). Points represent measured blood levels and line represents best fit line determined by nonlinear regression analysis.

to be 150 ng·mL^{-1}·h and 2.4 h, respectively. In a second identical experiment in the dog, essentially identical blood levels were measured, and the area under the curve was calculated to be 175 ng·mL^{-1}·h with a 2.7-h terminal elimination half-life.

It is important to point out that the cyclohexanone pyrolysis product of DQHS is not formed when authentic desoxyartemisinin is chromatographed under identical conditions for quantitation of DQHS. These results suggest that the mechanisms for the formation of the cyclohexanone pyrolysis product and desoxyartemisinin are unrelated, i.e., desoxyartemisinin is not a precursor of the cyclohexanone pyrolysis product, and also indicate that the presence of desoxyartemisinin in blood would not interfere with DQHS quantitation. Furthermore, desoxyartemisinin is thermally stable under mild GC conditions (injector, 180 °C; 15 M SPB-5; 100–200 °C at 10 °C/min) and can be detected and quantitated in blood under identical conditions of extraction as described above for DQHS. The presence of desoxyartemisinin in human blood samples after treatment with derivatives of QHS is anticipated, since this compound has been reported to be a metabolite of QHS in man (*10*).

Although there is not close structural similarity between the internal standards cedrol or triphenylmethanol and DQHS, the results reported in this study demonstrate that quantitation of DQHS in blood using this method is accurate and reproducible in the low to high nanogram per milliliter range and also show that this method can be utilized for pharmacokinetic studies at therapeutic doses in animals and man. Furthermore, much better accuracy, precision, and sensitivity should be obtained with a deuteriated internal standard; however synthesis of DQHS is currently extremely difficult due to the complex stereochemistry of the molecule. The method as described above currently has detection limit of 2 ng/mL with 5% of the *n*-butyl chloride extract on column. Since extraction of blood with *n*-butyl chloride results in much cleaner extracts and much higher recovery of DQHS even in the 2–20 ng/mL range, this change in the method results in lower detection limits, less interfering peaks, and longer

column lifetime. As shown in Figure 6 and also in animal pharmacokinetic studies currently in progress, this methodology is proving to be quite useful and will be utilized for pharmacokinetic studies in the clinic and aid in the development of this potent class of compounds for treatment of malaria in man.

The analysis of this class of compounds in biological fluids in the low nanogram per milliliter range is a significant challenge in which little progress has been made in the past. The studies reported in this paper were initiated when it was observed that adequate sensitivity could not be obtained by HPLC with reductive electrochemical detection (*14*). Unlike the HPLC method in which qinghaosu and any derivatives with the peroxide moiety can be detected and quantitated, this method was specifically designed for the analysis of DQHS after administration of ARTA, or other DQHS derivatives with a carboxyl moiety. Since ARTA and other ester or ether derivatives of DQHS also pyrolyze to the cyclohexane pyrolysis product, a carboxyl moiety permits separation of DQHS from the parent compound by liquid–liquid extraction. Therefore, this method is not useful for the analysis of DQHS formed from artemether or arteether (methyl or ethyl ethers of DQHS). This difficulty represents the major limitation in the general utility of the method for the analysis of DQHS; however, as shown in this paper, the method is useful for the quantitation of DQHS after administration of ARTA. Other studies to find a generally applicable method are in progress in this laboratory. In the interim, this method is now being used to study the pharmacokinetics of DQHS formation and elimination in various animal models.

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